Biosynthesis of phenylalanine and tyrosine in the methylotrophic actinomycete amycolatopsis methanolica

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Chapter 6

CHORISMATE MUTASE AND 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE OF THE METHYLOTROPHIC ACTINOMYCETE *AMYCOLATOPSIS METHANOLICA*

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Chorismate mutase (CM) and 3-deoxy-D-*arabino*-heptulosonate synthase (DS) are key regulatory enzymes in L-Phe and L-Tyr biosynthesis in *Amycolatopsis methanolica*. At least two CM proteins, CMIa and CMIb, are required for the single CM activity in wild-type. Component CMIa (a homodimeric protein with subunit M_r of 16,000) was purified to homogeneity (2,717 fold) and kinetically characterized. The partially purified CMIb preparation obtained also contained the single DS (DSI) activity detectable in wild-type. The activities of CMIa plus CMIb was inhibited by both L-Phe and L-Tyr. DSI activity was inhibited by L-Trp, L-Phe, and L-Tyr. A leaky L-Phe-requiring auxotroph, mutant strain GH141, grown under L-Phe limitation, possessed additional DS (DSII) and CM (CMII) activities. Synthesis of both CMII and DSII was repressed by L-Phe. An *ortho*-DL-fluorophenylalanine-resistant mutant of the wild-type (strain oFPHE83) that had lost the sensitivity of DSII and CMII synthesis to L-Phe repression was isolated. DSII was partially purified (M_r 42,000); its activity was strongly inhibited by L-Tyr. CMII was purified to homogeneity (93.6 fold) and characterized as a homodimeric protein with subunit M_r of 16,000, completely insensitive to feedback inhibition by L-Phe and L-Tyr. The activity of CMII was activated by CMIb; the activity of CMII plus CMIb was again inhibited by L-Phe and L-Tyr. A tightly blocked L-Phe-plus L-Tyr-requiring derivative of mutant strain GH141, GH141-19, that had lost both CMIa and CMII activities was isolated. The above-described properties, and the N-terminal amino acid sequences, showed that CMIa and CMII are one and the same protein.

INTRODUCTION

The aromatic amino acids L-Phe, L-Tyr, and L-Trp are synthesized via a common pathway. Erythrose-4-phosphate and phospho*enol*pyruvate are condensed into 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) by DAHP synthase (DS). DAHP is converted via the shikimate pathway into chorismate, involving six enzyme steps (Dewick, 1989). Chorismate mutase (CM) synthesizes prephenate from chorismate; this synthesis is an important and committed step in L-Phe and L-Tyr biosynthesis and is widespread in nature (Bentley, 1990). Chorismate is also converted into anthranilate (L-Trp biosynthesis), 4-aminobenzoate (folic acid biosynthesis), 4-hydroxybenzoate (ubiquinone biosynthesis) or isochorismate (menaquinone and enterobactin biosynthesis) (Bentley, 1990).

Aromatic amino acid biosynthesis is generally controlled by feedback inhibition or repression at the level of DS and CM. These proteins are present either as monofunctional or bifunctional (iso)enzymes, or as part of multienzyme complexes (Haslam, 1993).

Current knowledge on the biochemistry and regulation of the pathways of primary metabolism in actinomycetes (Gram-positive soil bacteria) is limited but considered to be important for further rational improvement of strains overproducing aromatic amino

acids and derived compounds (Bushell, 1989; Kell *et al.,* 1989). Many secondary metabolites synthesized by actinomycetes are derived from the aromatic amino acids themselves or from intermediates in their biosyntheses (Demain, 1983; Martin and Liras, 1982). Examples are the antibiotics rifamycin, vancomycin, and avoparcin, which are produced by the industrial actinomycete strains *Amycolatopsis mediterranei*, *Amycolatopsis orientalis*, and *Amycolatopsis coloradensis*, respectively (Embley, 1991; Gygax *et al.,* 1990; Haslam, 1993; Labeda, 1995).

We have initiated studies on glucose, methanol, and aromatic amino acid metabolism in the related methylotrophic actinomycete *Amycolatopsis methanolica* (De Boer *et al.,* 1990a; Warwick *et al*., 1994) and have purified and characterized several enzymes of glucose and quinate metabolism, prephenate dehydratase, and the multiple aromatic aminotransferases present in this organism (Abou-Zeid *et al.,* 1995; Alves *et al.,* 1994; Euverink *et al.,* 1992, 1995c). Previously we have also shown that the single DS (DSI) detectable in wild-type *A. methanolica* has the unique property of being feedback inhibited by all three aromatic amino acids (De Boer *et al.,* 1989). In this paper we report a detailed biochemical analysis of the DS and CM enzymes of *A. methanolica.*

MATERIALS AND METHODS

Microorganisms and cultivation

The *Amycolatopsis methanolica* wild-type strain (NCIB 11946) (De Boer *et al.,* 1990a), the plasmid pMEA300 (Vrijbloed *et al.,* 1994) deficient strain WV2 (Vrijbloed *et al.,* 1995), and auxotrophic mutants derived from strain WV2 (this study) were used. The procedures followed for the cultivation in batch cultures, harvesting of cells, and measurements of growth have been described previously (De Boer *et al.,* 1988). Glucose (1 M) was heat sterilized, and amino acid supplements were filter sterilized.

Mutant isolation

Mutants blocked in aromatic amino acid biosynthesis were isolated following UV irradiation treatments (Euverink *et al.,* 1992, 1995b).

Isolation of L-Phe analog-resistant mutants

Mutants of the wild-type strain resistant to the toxic L-Phe analog *ortho*-fluoro-DL-phenylalanine (oFPhe, 27.3 mM) were isolated on 10 mM glucose mineral agar (1.5% (w/v)) plates containing filter-sterilized analog. Fifteen agar plates were inoculated with approximately 5×10^7 cells each. After two weeks the spontaneous oFPhe-resistant colonies that had appeared were purified on homologous media.

Preparation of extracts and enzyme assays

Washed cell suspensions were disrupted in a French pressure cell at 140 MPa. Unbroken cells and debris were removed by centrifugation at 40,000 *g* for 30 min at 4°C. Following desalting through

PD 10 Pharmacia columns, the supernatant, containing 10-20 mg protein.ml^{-1}, was used for enzyme assays.
CM (EC 5.4.99.5) was assayed by measuring the amount of prephenate formed after conversion to phenylpyruvate (Dopheide *et al.*, 1972). The reaction mixture contained (100 µl) 50 mM Tris-HCl, pH 7.5, 2.0 mM chorismate, and extract or protein as indicated in the individual experiments. After 10 min, 10 µl 4.5 M HCl was added and the reaction mixture was incubated for 15 min at 37°C. The phenylpyruvate formed was estimated by adding 890 µl of an 1.58 M NaOH solution and measuring the A_{320} (ε_{320}[phenylpyruvate]= 17.5×10^3 M^{-1}.cm^{-1}). Endpoint measurements were indicative for initial reaction rates.
DS (EC 4.1.2.15) was assayed at 37°C by measuring the amount of DAHP formed from erythrose-4-phosphate and phospho*enol*pyruvate (De Boer *et al.*, 1989; Jensen and Nester, 1966a).

Purification of CMI from strain WV2

All chromatographic steps were carried out with a System Prep 10 liquid chromatography system (Pharmacia LKB Biotechnology Inc.).
Step 1. Glucose-grown cells (25 g wet weight) were harvested in the late exponential phase of growth. Extracts were prepared as described above in 25 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol (buffer A). DNase I (grade II, from bovine pancreas) and 1 mM $MgCl_2$ were added to the extract, and the mixture was incubated for 10 min.
Step 2. Hydrophobic interaction chromatography. The extract was adjusted to 1.1 M $(NH_4)_2SO_4$. Precipitated proteins were removed via centrifugation (15 min at 40,000 *g*). The resulting supernatant was applied to a column of butyl-Sepharose fast-flow (1.6 × 20 cm, 4°C) equilibrated in buffer A containing 1.1 M $(NH_4)_2SO_4$. Bound protein was eluted with a 400 ml decreasing linear gradient from 1.1-0.3 M $(NH_4)_2SO_4$ (flow rate, 4.0 ml.min^{-1}; fractions, 4.0 ml).
Step 3. Gel filtration chromatography. The protein from step 2 was concentrated by slowly adding solid $(NH_4)_2SO_4$ to 50% saturation. The mixture was stirred for 30 min at 4°C and centrifuged for 15 min at 40,000 *g*. The pellet was dissolved in 3 ml of buffer A and applied to a Superdex 200 (XK 16/60) gel filtration column previously equilibrated in buffer A containing 0.15 M KCl (flow rate, 1 ml.min^{-1}; fractions, 2 ml).
Step 4. Anion-exchange chromatography. The protein from step 3 was dialysed against 50 mM Tris-HCl, pH 8.8 containing 1 mM dithiothreitol (buffer B) and was applied to a Mono Q (HR 5/5) anion-exchange column. Bound protein was eluted with a 30 ml linear increasing gradient from 0-0.5 M KCl in buffer B (flow rate, 1 ml.min^{-1}; fractions, 0.5 ml).

Purification of CMII from mutant strain GH141

Step 1. Glucose-grown cells (25 g wet weight) were harvested in the late exponential phase of growth and an extract was prepared as described above in buffer A. DNase I (grade II, from bovine pancreas) and 1 mM $MgCl_2$ were added to the extract and, the mixture was incubated for 10 min.
Step 2. $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was slowly added to the extract to 35% saturation. The mixture was stirred for 30 min at 4°C and centrifuged for 15 min at 40,000 *g*. The resulting supernatant was adjusted to 50% saturation by adding solid $(NH_4)_2SO_4$. The mixture was stirred for 30 min at 4°C and centrifuged for 15 min at 40,000 *g*. The pellet was dissolved in 8 ml of buffer B and dialysed against the same buffer.
Step 3. Gel filtration chromatography. The protein from step 2 was applied to a Superdex 200 (XK 16/60) gel filtration column previously equilibrated in buffer B containing 0.15 M KCl (flow rate, 1 ml.min^{-1}; fractions, 2 ml).
Step 4. Anion-exchange chromatography. The protein from step 3 was dialysed against buffer B and was applied to a Mono Q (HR 5/5) anion-exchange column. Bound protein was eluted with

a 30 ml linear increasing gradient from 0-0.5 M KCl in buffer B (flow rate, 1 ml.min^{-1}; fractions, 1 ml).
Step 5. Hydrophobic interaction chromatography. The protein from step 4 was adjusted to 1.5 M $(NH_4)_2SO_4$ and applied to a phenyl-Superose (HR 5/5) column. Bound protein was eluted with a 20 ml linear decreasing gradient from 1.5-0 M $(NH_4)_2SO_4$ in buffer B (flow rate, 0.5 ml.min^{-1}; fractions, 0.5 ml).

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli and Favre (1973), with the marker proteins from the Combithek calibration protein kit (Boehringer Mannheim, Germany). Gels were stained with Coomassie brilliant blue R250.

Estimation of molecular weight

The M_r's were estimated by using a Superdex 200 column (XK 16/60) and Bio-Rad (Richmond, Calif., USA) gel filtration standards.

Kinetic studies

Kinetic parameters were determined at 37°C in 50 mM Tris-HCl, pH 7.5 and were calculated with the use of Sigmaplot for Windows 2.0 (Jandell Scientific Software) by using curve fitting with the Michaelis-Menten equation and its derivatives for various types of inhibition. Substrate and effector concentrations were as shown in the individual experiments.

Automated amino acid sequence determination

The CMIa and CMII proteins were applied to a Pro-spin cartridge (Applied Biosystems, Warrington, United Kingdom) containing a polyvinylidene difluoride membrane. Sequencing was performed on an Applied Biosystems Model 475A/120A automated gas-phase sequencer equipped with on-line high-pressure liquid chromatography (HPLC) for detection of phenylthiohydantoin amino acid derivatives (Eurosequence bv, Groningen, The Netherlands).

Analytical methods

Protein concentrations were determined with the Bio-Rad protein determination kit, with bovine serum albumin as the standard (Bradford, 1976). Glucose concentrations were determined with the GOD-period kit from Boehringer. Amino acid concentrations were determined by HPLC analysis (Euverink *et al.*, 1995c).

Biochemicals

Chorismate was obtained as barium salt from Sigma. Before use, barium ions were removed via precipitation with excess K_2SO_4. Chorismate was further purified on a Supelcosil LC-18-DB semi prep (5 µm) column (1.0 × 20.0 cm; Supelco, Inc., Bellefonte, Pa, USA) (Connelly and Siehl, 1987). All other chemicals were analytical grade and commercially available.

RESULTS

CM and DS in *A. methanolica*

CM and DS activities in extracts of wild-type *A. methanolica* WV2 were 7 and 22 mU.mg protein^{-1}, respectively. CM was inhibited by both L-Phe and L-Tyr, and DS was inhibited by all three aromatic amino acids (Table 1). The addition of L-Phe, L-Tyr, or L-Trp (100 mg.l^{-1} each), separately or in various combinations, to the growth medium of *A. methanolica* WV2 had no effect on the specific activities of CM and DS and feedback inhibition patterns.

Purification of CM from wild-type strain WV2

Purification of CM from *A. methanolica* WV2 turned out to be rather difficult. In a first attempt extract was applied to a Q-Sepharose anion-exchange column. Bound protein

Table 1. Specific activities of DS and CM in extracts of glucose-grown cells of *A. methanolica* WV2, and mutants harvested in late exponential phase of growth.

	Specific activity (U. mg protein^{-1})[a]			
	CM		DS	
Strain	alone	with L-Phe + L-Tyr	alone	with L-Trp
WV2[b]	7	1	22	2
GH141	43	42	50	30
GH141[c]	7	1	25	3
GH141-19[d]	0	0	22	4
oFPHE83	33	13	188	114
oFPHE83[c]	32	11	246	162

[a] CM activity was determined without effectors and in the presence of 1 mM L-Phe plus 1 mM L-Tyr. DS activity was determined without effectors and in the presence of 1 mM L-Trp.

[b] CM is also inhibited by 1 mM L-Phe (59%) and 1.0 mM L-Tyr (50%) separately. DS is inhibited not only by L-Trp (90% at 1.0 mM) but also by L-Phe (74% at 1.0 mM) and L-Tyr (60% at 1.0 mM) separately (De Boer *et al*., 1989).

[c] Glucose-grown cells, supplemented with 100 mg.l^{-1} L-Phe.

[d] Glucose-grown cells, supplemented with 100 mg.l^{-1} L-Phe plus 100 mg.l^{-1} L-Tyr.

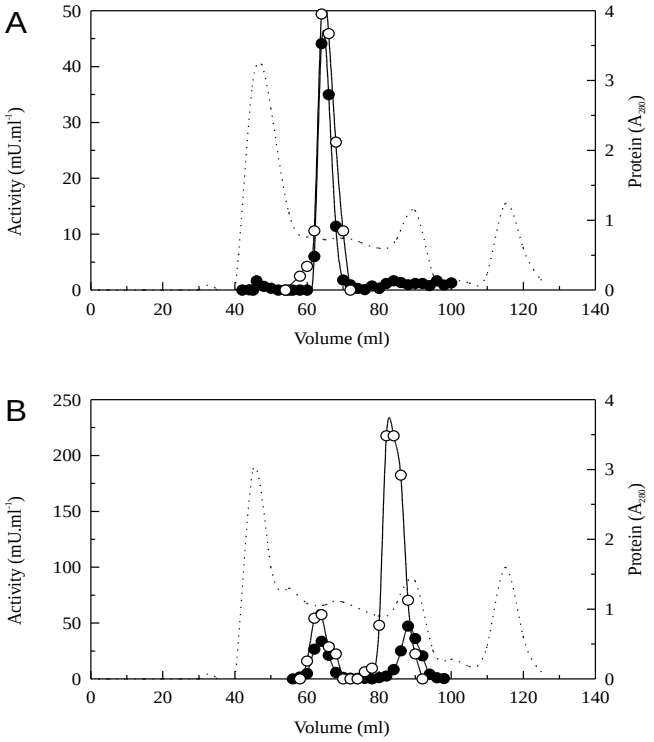

Figure 1. Superdex 200 gel filtration elution profiles of DS and CM in extracts of cells of *A. methanolica* WV2 (A, 21.9 mg protein) and mutant strain GH141 (B, 20.3 mg protein), grown in glucose mineral medium. ○, DS activity; ●, CM activity; ---, Protein (A_{280}).

was eluted with a linear gradient of 0-1 M KCl, but no activity was found. Hydrophobic interaction chromatography on butyl-Sepharose, however, yielded a single activity peak. Also, gel filtration of extracts revealed a single CM activity peak, coeluting with a single DS activity peak, corresponding to a M_r of 240,000 (Fig. 1A). In addition, dialysis against 50 mM Tris-HCl, pH 7.5 or pH 8.8 with or without 1 mM EDTA, centrifugation steps (40,000-100,000 *g*, 2 h) and ammonium sulphate precipitation did not inactivate CM. Small, diffusible effector molecules, e.g., metal ions or metabolites, thus are not required for CM activity. We subsequently observed that after Q-Sepharose anion-exchange chromatography CM activity could be restored by mixing the flowthrough (component CMIa) with fractions eluting from the column at approximately 0.3 M KCl (component CMIb). DS activity coeluted with component CMIb. Gel filtration of CMIa and CMIb separately, followed by reconstitution of CM activity with the other component, showed that they eluted in fractions corresponding to M_r's of 31,000 and 160,000, respectively.

Table 2. Purification of CMI from glucose-grown cells of *A. methanolica* WV2.

Step	Volume (ml)	Protein (mg)	Total activity (U)[a]	Specific activity ($U.mg^{-1}$)	Purification (fold)	Yield (%)
Extract	44	1562	8.3	0.005	1.0	100.0
Butyl-Sepharose	38	47.9	2.0	0.042	7.9	24.2
Superdex 200	8	13.0	1.2	0.091	17.0	14.2
Mono Q[b]	1	0.03	0.4	14	2717.0	5.2

[a] One unit of activity is defined as 1 µmol prephenate formed per minute from chorismate.

[b] Excess CMIb was added to assay the chorismate mutase activity of CMIa.

In this step DS also coeluted with component CMIb (M_r of 160,000), instead of behaving as a protein with a M_r of 240,000 (see above). Gel filtration of extracts (in the presence of up to 1 M KCl at pH 7.5) and hydrophobic interaction chromatography did not affect the interaction between CMIa and CMIb, showing that their binding is relatively strong. Gel filtration of extracts at pH 8.5 and 0.15 M KCl did result in separation of the CMIa and CMIb components.

Component CMIa, a minor protein in *A. methanolica*, was purified (2,717 fold) to homogeneity in four steps, with an overall yield of 5.2 % (Table 2). The purification protocol was designed in such a way that CM became separated into components CMIa and CMIb in the last step in the purification scheme only, eluting at 0.05 and 0.35 M KCl, respectively. In this way CM activity could be monitored more conveniently and homogeneous preparations of CMIa were obtained more easily. CM and DS activity coeluted during purification; in the final step DS coeluted with CMIb. The CMIb preparation was not subjected to further purification. SDS-PAGE of CMIa revealed a single band migrating at M_r 16,000. In view of its native M_r of 31,000, CMIa appears to be a homodimeric protein with a subunit M_r of 16,000.

The identification of N-terminal amino acids of CMIa was hampered by the low signal-to-noise ratio. The following seven N-terminal amino acids were tentatively identified: X, X, Q, X, N, E, K, L, T, and P (X, not identified). A BLASTP (Altschul *et al.*, 1990) search of the available databases revealed no significant homology with any other proteins described.

Kinetic studies were carried out with 0.3 µg of protein of pure component CMIa and 20 µg of the CMIb preparation (Fig. 2; Table 2). Under these conditions, CMIa is clearly limiting for the overall CM activity (see inset Fig. 2). The K_m for chorismate was 2.0 ± 0.3 mM (mean ± standard deviation). The calculated V_{max} value was 47 ± 2.9 U.mg

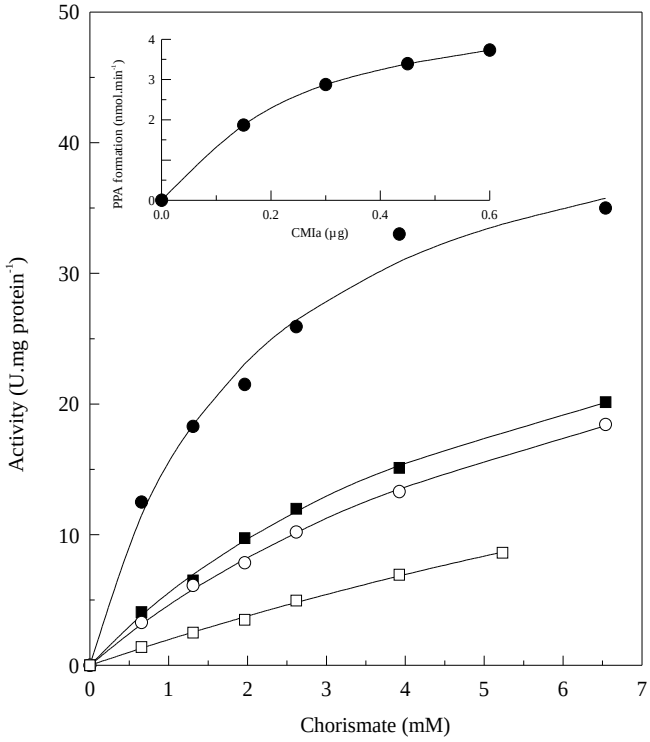

Figure 2. Specific CM activity of component CMIa (0.3 µg of protein) plus excess of component CMIb (20 µg of protein) with increasing chorismate concentrations in the absence of an effector (●), or in the presence of 2.0 mM L-Phe (■), 2.0 mM L-Tyr (○), and 2.0 mM L-Phe plus 2.0 mM L-Tyr (□). (Inset) CM activity with increasing concentrations of CMIa in the presence of 20 µg of protein of preparation CMIb at a chorismate concentration of 2.0 mM (PPA, prephenate).

protein^{-1}. CM activity was competitively inhibited by L-Phe and/or L-Tyr. The K_i values for L-Phe and L-Tyr were 0.66 ± 0.02 mM and 0.52 ± 0.01 mM, respectively.

Characterization of L-Phe auxotrophic mutants

A leaky L-Phe auxotrophic mutant, strain GH141, which had lost 90% of the L-Phe aminotransferase activity was isolated previously (Abou-Zeid *et al.*, 1995). Cultivation of the strain in glucose mineral medium showed that the doubling time of strain GH141 had increased to 6 h, because growth had become limited by the rate of L-Phe biosynthesis. The doubling time of mutant strain GH141 in glucose mineral medium supplemented with L-Phe (100 mg.l^{-1}) was comparable to that of the wild-type (2.5 h). Extracts of mutant

strain GH141 cells grown in glucose mineral medium displayed DS and CM activities at about 2.5 to 6 times higher than those of the wild-type. In mutant strain GH141, CM activity was not feedback inhibited by either L-Phe or L-Tyr, and the sensitivity of DS to L-Trp was strongly reduced (Table 1). Fractionation of extracts of mutant strain GH141, grown on glucose mineral medium, on a Superdex 200 gel filtration column revealed additional CM (CMII, M_r 31,000) and DS (DSII, M_r 42,000) activity peaks (Fig. 1B). The CMI and DSI enzymes in wild-type and in mutant strain GH141 possessed identical molecular weights and feedback inhibition sensitivities. Cells of strain GH141 grown in glucose mineral medium supplemented with 100 $mg.l^{-1}$ L-Phe displayed wild-type levels of CM and DS activities and properties (Table 1) and completely lacked the CMII and DSII activity peaks.

Strain GH141-19, a tightly blocked L-Phe- plus L-Tyr-requiring auxotrophic mutant, subsequently was derived from strain GH141. Strain GH141-19 had completely lost CM activity (Table 1). The addition of pure component CMIa to extracts of mutant GH141-19 restored CM activity to wild-type levels. This indicates that strain GH141-19 is deficient in component CMIa specifically.

Characterization of DSII

Further attempts to purify DSII after gel filtration chromatography failed because of a significant loss of activity, which occurred during anion-exchange or hydrophobic interaction chromatography. The addition of divalent cations to buffer solutions and mixing of fractions were not successful. Some characteristics of DSII therefore were determined after gel filtration. DSII remained active for at least 24 h at 4°C. The apparent K_m values for phospho*enol*pyruvate (3.8 mM erythrose-4-phosphate) and erythrose-4-phosphate (3.0 mM phospho*enol*pyruvate) were 0.40 ± 0.06 mM and 1.1 ± 0.2 mM, respectively. With 3.8 mM phospho*enol*pyruvate and 1.7 mM erythrose-4-phosphate, DSII activity was inhibited by 1.0 mM L-Trp (17%) and 1.0 mM L-Tyr (83%) but not by L-Phe. L-Tyr inhibition was competitive with respect to erythrose-4-phosphate concentration, and the K_i value for L-Tyr was 0.031 ± 0.006 mM. Noncompetitive inhibition by L-Tyr was found with respect to phospho*enol*pyruvate concentration, and the K_i value was 0.8 ± 0.2 mM. Intermediates in the biosynthesis of aromatic amino acids (shikimate, anthranilate, chorismate and prephenate; 1 mM each) inhibited DSII activity for less than 15%.

Table 3. Purification of CMII from glucose-grown cells of *A. methanolica* mutant strain GH141.

Step	Volume (ml)	Protein (mg)	Total activity (U)[a]	Specific activity ($U.mg^{-1}$)	Purification (fold)	Yield (%)
Extract	49	882.0	47.5	0.05	1.0	100.0
Butyl-Sepharose	8	2.5	50.3	0.13	2.5	105.9
Superdex 200	24	4.6	21.6	0.25	4.6	45.5
Mono Q	4	69.5	17.7	3.75	69.5	37.2
Phenyl-Superose	2	2.3	11.7	5.05	93.6	24.6

[a] One unit of activity is defined as 1 µmol prephenate formed per minute from chorismate.

Characterization of CMII

The CMII enzyme was much more abundant than CMIa in *A. methanolica*. CMII was purified (93.6 fold) to homogeneity from cells of mutant strain GH141 with an overall yield of 25% (Table 3). CMII bound to a Mono Q anion-exchange column at pH 8.8, but not at pH 7.5, and eluted at approximately 0.05 M KCl. SDS-PAGE of CMII revealed a single band migrating at M_r 16,000. In view of its native M_r of 31,000, CMII appears to be a homodimeric protein with subunit M_r of 16,000.

The following 22 N-terminal amino acids were identified: M, A, Q, T, N, E, K, A, T, P, X, E, T, S, G, E, P, V, A, S, A, X, E, and I (X: not identified). A BLASTP (Altschul *et al.*, 1990) search of the available databases revealed no homology with other CM enzymes described. Six of seven amino acids, however, were identical to tentatively identified N-terminal amino acids of component CMIa (see above).

A virtual linear relation was observed between the chorismate concentration, in the range of 0 - 4 mM, and CMII activity (Fig. 3). L-Phe and L-Tyr (1 mM concentrations) did not inhibit CMII activity or modify this linear relation (data not shown). CMII was activated by CMIb, resulting in Michaelis-Menten kinetics (see inset Fig. 3). The K_m value for chorismate was 2.2 ± 0.2 mM and a V_{max} of 47 ± 2.5 U.mg protein^{-1} could be calculated. The activity of CMII plus CMIb was inhibited by L-Phe and L-Tyr (Fig. 3), albeit less strongly than that of CMIa plus CMIb (Fig. 2). CMII plus CMIb activity was competitively inhibited by L-Phe and/or L-Tyr. The K_i values for L-Phe and L-Tyr were 4.5 ± 0.4 mM and 3.8 ± 0.5 mM, respectively.

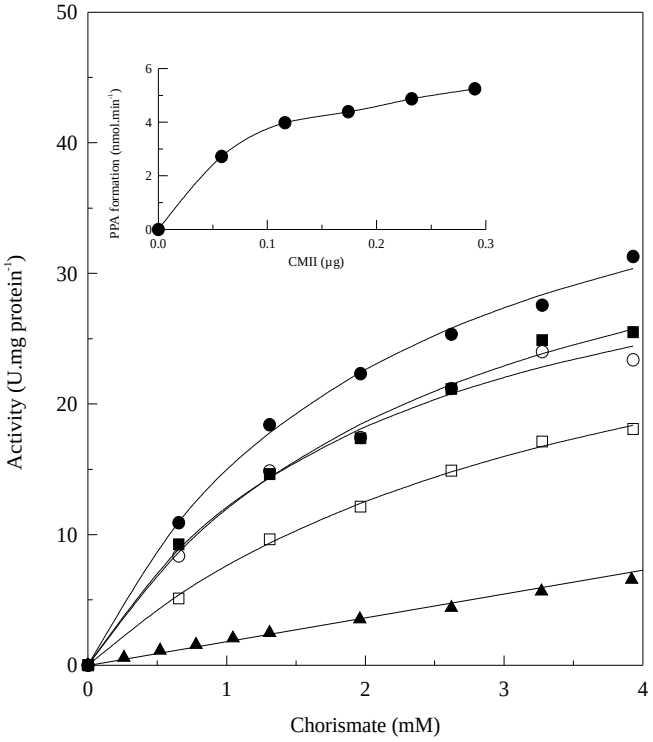

Figure 3. Specific CM activity of CMII (1.2 µg of protein) (▲) and CMII (0.23 µg of protein) plus excess of component CMIb (20 µg protein) with increasing chorismate concentrations in the absence of an effector (●), or in the presence of 2.0 mM L-Phe (■), 2.0 mM L-Tyr (○), and 2.0 mM L-Phe plus 2.0 mM L-Tyr (□). (Inset) CM activity with increasing concentrations of CMII in the presence of 20 µg of protein of preparation CMIb at a chorismate concentration of 2.0 mM (PPA, prephenate).

Isolation and characterization of mutant strain oFPHE83

De Boer *et al*. (1990c) provided evidence that the toxic L-Phe analog oFPhe blocks growth of *A. methanolica* in glucose mineral medium via prephenate dehydratase or CM inhibition. In the present study we observed that oFPhe-resistant mutant strains of wild-type *A. methanolica* (NCIB 11946) could be isolated readily. After two weeks of incubation, numerous spontaneous oFPhe-resistant colonies were clearly visible against a background of tiny colonies. Initially, a total of 400 colonies resistant to oFPhe were selected. After repeated transfers, 122 oFPhe resistant mutants still scored clearly positive. The CM enzymes of five oFPhe-resistant strains with normal growth rates in glucose mineral medium were analysed for their sensitivity towards feedback regulation. In four strains CM and DS displayed wild-type levels of activities and degrees of

sensitivity for feedback regulation. Mutant strain oFPHE83 possessed (very) high levels of CM and DS activities (Table 1). Fractionation of extracts of mutant strain oFPHE83 via gel filtration revealed the additional presence of DSII and CMII enzymes (data not shown). Unlike the situation in the wild-type and in mutant strain GH141, the synthesis of the DSII and CMII enzymes was not repressed by L-Phe in mutant strain oFPHE83 (Table 1). The CMIa and CMII enzymes and the DSI and DSII enzymes in the wild-type, mutant strain GH141, and in strain oFPHE83 possessed otherwise identical molecular weights and feedback inhibition sensitivities.

DISCUSSION

The limited studies thus far carried out with Gram-positive bacteria have revealed the presence of isoenzymes of CM in bacilli only (Gray *et al.*, 1990b; Lorence and Nester, 1967; Nasser *et al.*, 1969). In *Bacillus subtilis* one of these CM isoenzymes constitutes a bifunctional protein with DS (Nakatsukasa and Nester, 1972). A CM-DS enzyme complex has also been reported for *Brevibacterium flavum* (Shiio and Sugimoto, 1979, Sugimoto and Shiio, 1980a, 1980b). This paper is the first report of a complex of DS and CM in an actinomycete. CM activity in wild-type *A. methanolica* requires at least two proteins (CMIa and CMIb), and in several purification steps coelution of the DS (DSI) and CM activities occurred (this study). In the final step of the purification protocol for component CMIa, DSI coeluted with CMIb. Also, the molecular weights of DSI (M_r 168,000, De Boer *et al.*, 1989) and CMIb (M_r 160,000) are similar. These data thus suggest that CMIb and DSI are identical. Considering the molecular weights of components CMIa and CMIb-DSI, two CMIa dimers (M_r 31,000) are most likely associated with CMIb-DSI in a complex with a M_r of 240,000 (Fig. 1A).

CM activity in *A. methanolica* is feedback inhibited by L-Phe and L-Tyr, a characteristic that is shared with the enzyme from *A. mediterranei* (Xia and Chiao, 1989). The *Corynebacterium glutamicum* (Hagino and Nakayama, 1975) and *B. flavum* (Sugimoto and Shiio, 1980b) CM enzymes were also inhibited by L-Phe and L-Tyr; this inhibition was released in the presence of L-Trp, a characteristic not shown by the *A. methanolica* CM. The sporeforming members of the order *Actinomycetales* display CM activity that is inhibited by L-Tyr and/or L-Trp only (Hund *et al.*, 1987; Speth *et al.*, 1989). The CM of *Streptomyces aureofaciens* contains at least three subunits with a M_r of 14,000; the enzyme is insensitive to inhibition by aromatic amino acids (Görisch, 1987). Also, the CM (M_r 75,000) detected in extracts of *Streptomyces* sp. strain 3022a, a chloramphenicol producer, was not inhibited by aromatic amino acids (Lowe and Westlake, 1972).

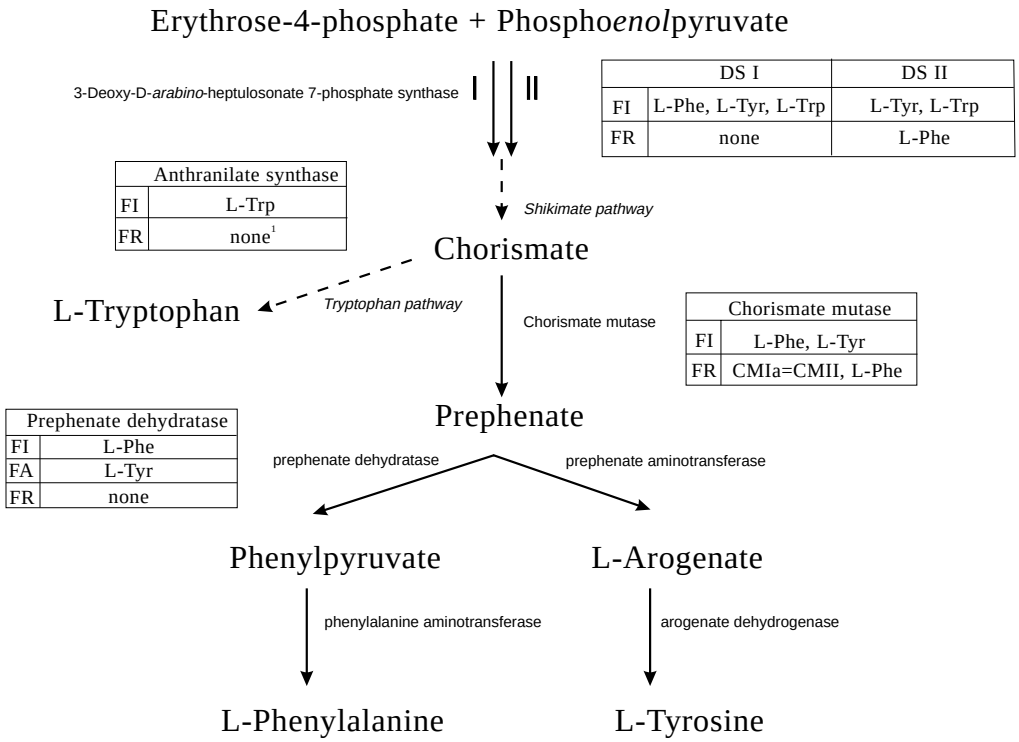

Figure 4. Regulation of aromatic amino acid biosynthesis in *A. methanolica*. FI: feedback inhibition; FA: feedback activation; FR: feedback repression; dashed lines: multiple enzyme steps; none[1], data from De Boer *et al.* (1989).

Characterization of the leaky L-Phe aminotransferase mutant strain GH141 (Abou-Zeid *et al.*, 1995) revealed that synthesis of a second CM (CMII) and a second DS (DSII) became derepressed under L-Phe limiting conditions. Following growth in the presence of L-Phe, only the constitutive DSI with L-Trp as main negative effector and CMI with L-Phe and L-Tyr as the main negative effectors were present (Table 1). The analog-resistant mutant strain oFPHE83 most likely has lost L-Phe feedback repression sensitivity, resulting in enhanced DS and CM activities due to DSII and CMII derepression. L-Phe repression of CM (but not of DS) synthesis was also reported in *C. glutamicum* (Hagino and Nakayama, 1974b, 1975). In *B. flavum* both CM and DS syntheses are repressed by L-Tyr (Shiio and Sugimoto, 1979).

DSII is a protein with a M_r of 42,000 that is strongly feedback inhibited by L-Tyr and less strongly inhibited by L-Trp. De Boer *et al*. (1989) reported that the subunits with a M_r of 41,000 of the tetrameric DSI protein from *A. methanolica* may also display DS

activity. It appears unlikely, however, that subunits of DSI are responsible for DSII activity: L-Tyr strongly inhibits DSII but not the DSI subunits (Table 1). Thus, *A. methanolica* most likely possesses DSI and DSII isoenzymes. This also may explain why an extensive search for auxotrophic mutants of *A. methanolica*, yielded approx. 150 aromatic amino acid auxotrophs but not DS-negative mutants (Euverink *et al.*, 1995b).

The low M_r DS protein (DSII) detected in *A. methanolica* is unique in prokaryotes and has been reported for the yeast *Saccharomyces cerevisiae* (M_r 39,000 and 41,000) only (Braus, 1991). The only other actinomycete DS that has been described thus far is a relatively large, oligomeric protein of *Streptomyces rimosus* (Stuart and Hunter, 1993).

The respective native and subunit molecular weights of CMIa and CMII are equal; also the K_m values for chorismate and V_{max} values for CMIa plus CMIb and CMII plus CMIb are similar. These properties, and the N-terminal amino acid sequences, suggest that CMIa and CMII are identical proteins. This was confirmed by the isolation of the CM-deficient mutant strain GH141-19. A striking difference between CMIa and CMII, the activity of CMII alone, can be explained by the much higher concentrations of CMII present in extracts. When CMIa and CMII were used at similar low concentrations, no activity was detectable; the addition of CMIb restored activity in both cases. The differences in feedback inhibition sensitivity between the CM activity of CMIa plus CMIb and CMII plus CMIb for L-Phe and/or L-Tyr remain to be explained, however.

The N-terminal amino acids identified for the CMIa and CMII proteins did not show any significant similarity with other known proteins, suggesting that the *A. methanolica* enzyme may belong to a new class of CM enzymes. Cloning of the gene encoding CMIa-CMII is currently in progress. In future work we will study the unique regulatory properties of this actinomycete CM in more detail.

In conclusion, L-Phe and L-Tyr biosynthesis in wild-type *A. methanolica* is regulated via, (i) L-Phe inhibition and L-Tyr activation of prephenate dehydratase activity, (ii) L-Phe and L-Tyr inhibition of CM activity, and (iii) repression of CMIa-CMII and DSII synthesis by L-Phe (Fig. 4). Under L-Phe limiting conditions derepression of both CMIa-CMII and DSII synthesis increases the flux of intermediates toward L-Phe and L-Tyr biosynthesis. L-Tyr inhibition of DSII will prevent the accumulation of L-Tyr under L-Phe limitation (Fig. 4). The relative *in vivo* contribution of these control mechanisms remains to be determined.

REFERENCES

References are listed on pages 147-160.